

**LIGHT-INDUCED ABSORBANCE CHANGES IN ORGANS
AND MEMBRANE FRACTIONS FROM HIGHER PLANTS,
WITH A POSSIBLE CONNECTION TO THE BLUE LIGHT
PHOTORECEPTOR**

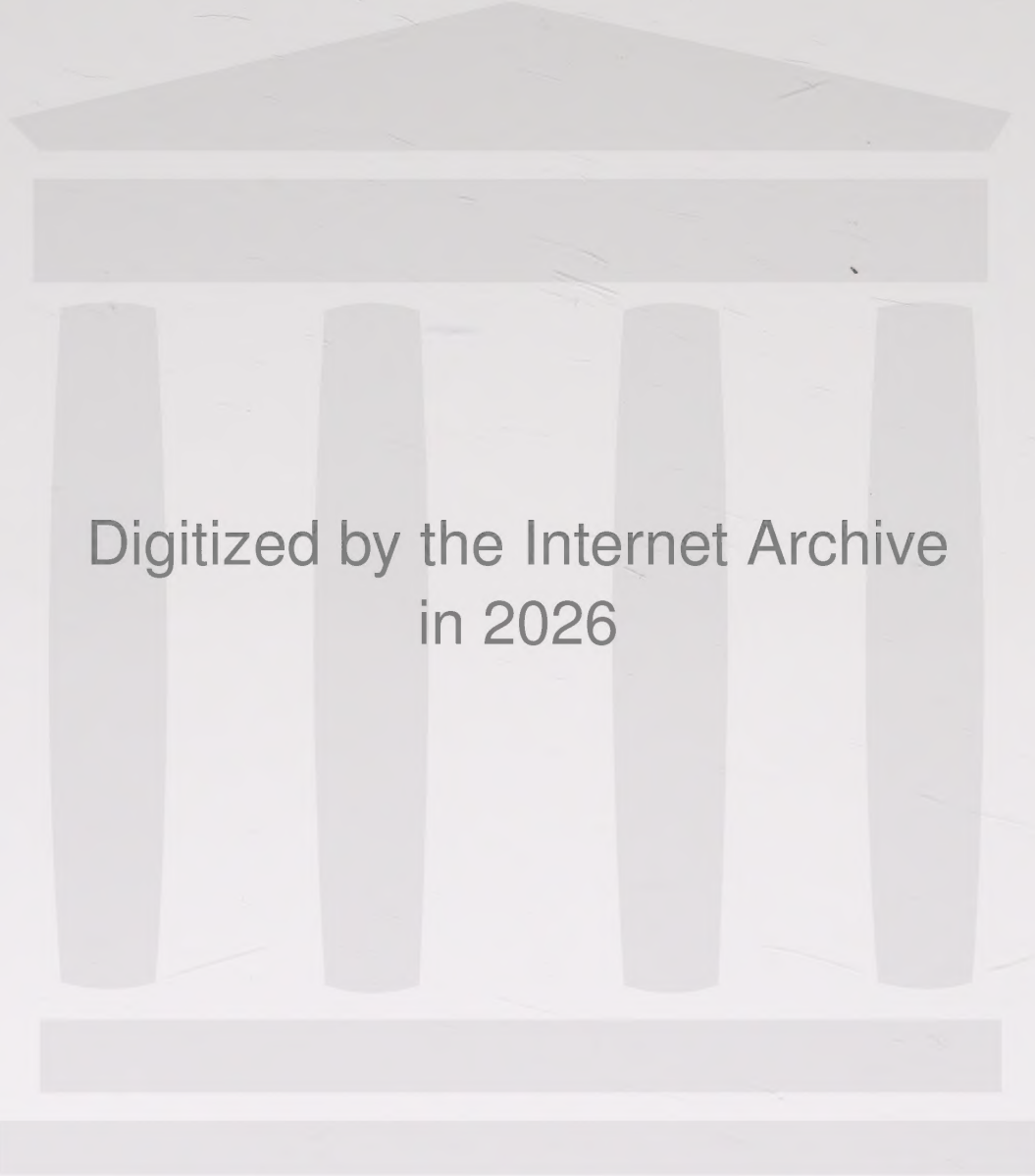
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DEPARTMENT OF PLANT PHYSIOLOGY

UNIVERSITY OF LUND, SWEDEN

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By

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1 INTRODUCTION

Light is important to plants, both as energy source for photosynthesis, and as a regulator of their development. Pigment synthesis, germination, movements and flowering are only a few examples of processes regulated by light. It was realized quite early that different spectral regions were unequally effective for a given process. In many cases, a red far-red reversible pigment, phytochrome, is involved, whereas in others, blue light (and UV) is needed.

Phototropism, i.e. the bending of an organ in relation to a light stimulus, is an intensively studied blue light and UV phenomenon, especially in grass coleoptiles and fungal sporangiophores. Still, there are several obscurities concerning the primary steps in the reaction. Basic findings in phototropism of coleoptiles are the following: At a low fluence, the coleoptiles bend towards the light (1st positive curvature). As the fluence is increased, more bending is obtained until a plateau is reached (for *Avena* coleoptiles, this corresponds to an energy of 0.1 J m^{-2} , of 436 nm light, Zimmermann and Briggs 1963). The results at even higher fluences depends on the irradiance used. At a low one (10^{-4} W m^{-2}) the bending starts to increase again with increasing fluence (2nd positive curvature), whereas at 100 times larger irradiances, the coleoptiles bend away from the light (1st negative curvature) prior to the second bending towards the light. Second negative as well as third positive curvatures can also be obtained under appropriate conditions. Reciprocity only holds for the first positive and negative phases (i.e. time can substitute for light only here). Pretreatment with red light increases the blue light fluence needed for the first positive curvature and decreases that needed for the third (which means that the second one more or less disappears).

The similarity in blue light (and UV) requirements for many developmental processes in plants and fungi with action

maxima at 365 and 450 nm and no action above 500 nm, has led to the postulate of a ubiquitous photosensitizer. Flavins and carotenoids have become candidates for such a role. Both groups are photochemically active; flavins as prosthetic groups of several light sensitive enzymes and carotenoids in the photosynthetic apparatus. Intensive research has been undertaken in order to discriminate between the two possibilities. Doubts have, however, been raised whether only one type of pigment molecule is involved in all blue light processes (Gressel 1979). Some enzymes are clearly regulated by FMN (e.g. glycolate oxidase, Schmid 1970). In *Neurospora*, light absorption by nitrate reductase (which contains FAD) induces conidiation, but does not cause phase shifts of the conidiation rhythm (Klemm and Ninnemann 1978). Carotenogenesis of this fungus has an action spectrum that is slightly different from the regular blue light processes and carotenoids have been proposed as photosensitizers in this case (DeFabo 1976). Later it has been suggested that the biphasic nature of the response to increasing fluence in carotenoid synthesis is due to two different photoreceptors, one, a carotenoid, working at low fluences, the other one, perhaps a flavin, working at higher fluences (Shropshire 1979). In other processes, e.g. phototaxis of the slime mold *Dictyostelium*, the absorbing pigment is a porphyrin (Poff and Butler 1974 b).

In the case of phototropism, however, most data point to a flavin as active component. Pill-Soon Song (1979) described a photochemically different flavin that was specifically located in coleoptilar tips. By the use of laser light, Delbrück et al. (1976) showed that phototropic and light growth responses in *Phycomyces* could be elicited after excitation with red light. This would correspond to a direct excitation of a flavin to its triplet state. Involvement of triplet flavin was also suggested by Schmidt et al. (1977) in their work with corn seedling phototropism. After addition of triplet quenchers such as iodide the sensitivity towards blue light was decreased. The same inhibition was

was obtained if phenyl acetic acid was added. This substance is known to bind to excited flavins. Red light, superimposed on the blue resulted in smaller bending than if the blue light was supplied alone. This was interpreted as being due to depopulation of the triplet state of a flavin.

Löser and Schäfer (1979) showed that red light could be effective in the phototropism of *Phycomyces*, and that it reversed the effect of blue if applied simultaneously. They drew the attention to the photochrome model in flavin photobiology, proposed by Hartmann (1978). He suggested that for flavin-mediated reactions, as in the case of the phytochrome-mediated HIR, the photostationary state determines the response at high fluence. The active species absorbs at 420 nm and could be identified as a flavin semiquinone and the inactive is the 450 nm absorbing species (Hertel, cited by Löser and Schäfer 1979).

Experience from the pigment responsible for sensitivity to red light, i.e. phytochrome, where the red far-red reversibility also could be detected spectrally as a shift of a wavelength band, led people to search for similar changes when blue light was delivered. The success came in the beginning of the seventies when two groups (Poff and Butler 1974 a, Muñoz et al. 1974) working with fungal material showed that blue light induced a rapid bleaching in the blue region, corresponding to a flavin reduction. It was followed by absorbance increases indicating a b-type cytochrome reduction. During subsequent darkness, oxidation of both pigments took part. It was not possible, however, to obtain any absorbance changes until the material had been metabolizing in the cuvette for a couple of hours. This indicates that reduced oxygen tension was needed for the absorbance changes to be observed. Similar blue light induced absorbance change (LIAC) were also obtained in corn coleoptile preparations (Briggs 1976). After fractionation of fungal mycelium (*Neurospora*) or corn coleoptiles most activity was found in a fraction sedimenting at an intermediary speed (21 000 g).

This corresponded to the distributions of NaATPase in the fungus, a plasma membrane located enzyme (Brain et al. 1977).

The present work was done in order to describe this LIAC related cytochrome in corn in more detail. Methylene blue has been used throughout as a substitute for the endogenous photoreceptor. The relevance with which this can be done, will be described. A purification procedure will be presented, also used with other plant materials. Light induced absorbance changes not correlated to this b-type cytochrome, but occurring under certain conditions, will be shown. Finally the physiological significance related to either of these light induced absorbance changes will be discussed.

This thesis is based on the following publications

- I Widell, S. & Björn, L.O. 1976. Light induced absorbance changes in coleoptiles. - *Physiol. Plant.* 36: 305-309.
- II Britz, S.J., Schrott, E., Widell, S. & Briggs, W.R. 1979. Red light-induced reduction of a particle associated b-type cytochrome in the presence of methylene blue. - *Photochem. Photobiol.* 29: 359-365.
- III Widell, S., Britz, S.J. & Briggs, W.R. Further characterization of the red light-induced reduction of a particle associated b-type cytochrome from corn in the presence of methylene blue. (Submitted.)
- IV Widell, S. 1980. The effect of detergent treatment on methylene blue sensitized cytochrome b photoreduction in fractions from corn coleoptiles. - *Physiol. Plant.* 48: 353-360.
- V Results which are published in this dissertation.

Abbreviations: 9 KP, 500 - 9 000 g, 20 min pellet; 21 KP, 9 000 - 21 000 g, 20 min pellet; 50 KP, 21 000 - 50 000 g, 45 min pellet; LIAC, light induced absorbance change (if not stated otherwise, the difference in ($A_{248}-A_{410}$), induced by 10 s light, is meant.); MB, methylene blue; MOPS, morpholinopropane sulfonic acid; DOC, deoxycholate, sodium salt; SDS, sodium deoxycholate; NADH, nicotinamide adenine dinucleotide, reduced, disodium salt; IDP, inosine 5'-diphosphate; EDTA, ethylenediamine tetraacetic acid; PEG, polyethylene glycol; $\Delta\Delta A$, absorbance difference change; UV, ultraviolet radiation.

2 EXPERIMENTAL METHODS

Caryopses from wheat (*Triticum aestivum* L. var Starke and Starke II, cv. Weibulls, Landskrona, Sweden), barley (*Hordeum vulgare* L. var Kristina, cv. Weibulls, Landskrona, Sweden) and corn (*Zea mays* L., WF 9 x Bear 38 Decatur, Illinois) were grown for 4-5 days in darkness at 25-27°C in covered plastic boxes. The corn obtained 2 h red light each night except the last to suppress mesocotyl growth. The seedlings were harvested under dim green light and the leaves inside the coleoptiles were discarded in order to minimize absorbance changes due to phototransformations of protochlorophyllide. The coleoptiles were then used directly (I) or prepared as described below. Cauliflower inflorescences (*Brassica oleracea*, purchased at the local market) were fractionated directly (see below).

The material was homogenized with mortar and pestle in a medium composed of 0.1 M MOPS, 3 mM EDTA, 0.1 mM MgCl₂, 0.25 M sucrose and 0.1 % (v/v) 2-mercaptoethanol; pH 7.4 except in V. The homogenate was differentially centrifuged 500 g, 10 min; 9 000 g, 20 min; 21 000 g, 20 min and 50 000 g, 45 min yielding the pelleted fractions 0.5 KP, 9 KP, 21 KP and 50 KP, respectively. The pellets were resuspended in the same medium as used in the homogenization step, except that 2-mercaptoethanol was omitted and pH adjusted to 7.0, and stored frozen (-18°C) until used. When the material was separated by means of aqueous two phase polymer systems, homogenization was performed with a Sorvall Omnimixer (DuPont Instruments) in a medium composed of 0.025 M MOPS, 0.325 M sucrose, 3 mM EDTA, 0.1 mM MgCl₂, 0.1 % (w/v) cysteine; pH 7.8. The homogenate was pelleted as above, except that 21 KP and 50 KP were collected together. The pellets were resuspended in 0.25 M sucrose and 5 mM potassium phosphate buffer; pH 7.8. This suspension was added to the phase system which had a final composition of 6.1 % (w/w) dextran T 500, 6.1 % (w/w) PEG 4 000, 0.25 M sucrose, 5 mM potassium phosphate buffer; pH 7.8. After mixing, partition was obtained with 2-4 min

centrifugation at 500 g. Each phase was collected, diluted and pelleted at 80 000 g, 1 h. The interphases were collected together with their respective bottom phases. The pellets were resuspended in the same resuspension medium as used above, and stored frozen (-18°C) until used. All preparation steps took place in dim green light except when cauliflower inflorescences were used, and at 5°C . In the detergent experiments, the material was judged as solubilized if not pelleted by 1 h centrifugation at 140 000 g.

The spectrophotometric measurements were performed with a dual wavelength spectrophotometer operated either in the split beam or in the dual wavelength mode. In the latter case, the wavelength pairs were chosen either so as to give maximal signal (maximum vs. minimum in a difference spectrum) or to show the changes at one wavelength compared to a nearly isosbestic reference point (Aminco DW-2 or Perkin Elmer 356). The actinic light was generated by a xenon lamp, either connected to a Bausch & Lomb High Intensity monochromator or equipped with RG 2 filters. This resulted in irradiances of $10\text{-}15\text{ W m}^{-2}$ and $20\text{-}40\text{ W m}^{-2}$, respectively. These irradiances are much larger than those applied to whole organs in experiments on phototropism with the accompanying complicated multiphasic result. The light hit the sample at right angles to the measuring beam. If spectrally possible, the photomultiplier was protected from the actinic light by means of glass filters. For further details, the reader is referred to I - V.

3 RESULTS AND DISCUSSION

3.1 Can an exogenous dye substitute for the endogenous photoreceptor in blue light induced photoreduction of a b-type cytochrome? (II and III)

This problem arose because a reproducible LIAC was difficult to obtain in fractions from higher plants (Brain et al. 1977). Therefore, a detailed study of the b-type cytochrome was difficult to make. Besides, a study of the reaction while the light was on was difficult since the actinic and measuring beams were not spectrally separated.

Photodynamic dyes have been used in many experiments as substitutes for endogenous photoreceptors. Sagromsky (1956) used methylene blue (MB) and used red light for excitation to enhance conidiation in the fungus *Trichotechium roseum*; a process normally governed by blue light (and UV) and endogenous photoreceptor(s). Lang-Feulner and Rau (1975) demonstrated that carotenogenesis in the fungus *Fusarium aquaeductum*, normally induceable by blue light (and UV), was induceable by red light treatment after the mycelia had been soaked in photodynamically active dye (MB, toluidine blue or neutral red) but not in photodynamically inactive dyes (malachite green or dichlorophenol indophenol). They interpreted this as being due to a photo-oxidation of a metabolite by the added dye. This was also consistent with the capacity of hydrogen peroxide to substitute for the light. Thus, the same result was obtained when exogenous dyes were used, as with the endogenous photoreceptor(s). The similarity does not necessarily hold as concerns the mechanism with which this result is obtained.

In the systems studied here, i.e. membrane fractions from corn coleoptiles, the addition of MB and subsequent irradiation with broad band red light (wavelength >600 nm) resulted in similar absorbance changes as those reported by others with blue light irradiation of fungi. This LIAC, with maxima

at 428 nm (Soret band) and 557 nm (α -band) and a minimum at 410 nm was enriched in the membrane fraction sedimenting at 21 000 g. Not more than 10-30 % of the cytochrome in any of the fractions 9 KP, 21 KP and 50 KP could accept electrons from photoreduced MB. In all fractions the fused Soret peak of total cytochrome was found at shorter wavelengths compared to the LIAC peak.

The 9 KP, which was enriched in mitochondria, had a relative LIAC activity (LIAC per mg protein) of one third of that in 21 KP, whereas the value for the 50 KP, enriched in endoplasmatic reticulum was two thirds. The data are similar to those obtained by Brain et al. (1977) for *Neurospora* preparations and the distribution of LIAC also corresponds to that of the low potential cytochrome in corn coleoptiles as described by Jesaitis et al. (1977). Moreover, in experiments with fractions from corn coleoptiles where the oxygen tension was kept lower (by means of a glucose-glucose oxidase system) reproducible blue light induced absorbance changes could be detected with similar distribution as those obtained here after red light irradiation in the presence of methylene blue (Goldsmith 1980). Finally, Brain (personal communication) has shown that in *Neurospora*, MB and red light can substitute for endogenous photoreceptor and blue light (and UV), respectively, in inducing LIAC as well as for developmental processes.

With a useful photosensitizer, saturation with respect to dye concentration and light should be reached, indicating that some kind of specificity is involved. When the dye concentration was increased, larger $\Delta(A_{428}-A_{410})$ was obtained in all fractions up to a point where further dye acted inhibitory. The pattern was quite complicated, probably due to dye-dye as well as dye-sample interactions (McKay 1968). Besides, at the higher concentrations used, light penetration was limited.

In the mitochondrial fraction, saturation was obtained at a higher MB concentration (100 μ M) than in 21 KP and 50 KP (50 μ M). This was probably due to light-induced redox changes

in cytochrome oxidase as well. In the 21 KP and 50 KP fractions, LIAC measured at low MB concentration (1 μM) was 2-3 fold larger if the samples were diluted to a quarter compared to non-diluted samples containing 2-3 mg protein/ml. This could be due to non specific attachment to other structures in the sample. Addition of urea, known to decrease such interactions as well as to split dimers of dyes (Mukerjee and Ghosh 1963) resulted in slightly increased LIAC at low concentrations of MB indicating that non specific interaction really took part. The maximal LIAC was not altered.

If, at a fixed MB concentration, the irradiance of a sample was increased, the steady state level of reduced cytochrome b increased hyperbolically. In the fluence interval tested (30-440 J m^{-2}) reciprocity did not hold. Since the excitation light obtainable was not strong enough, maximal LIAC ($\Delta\Delta A_{\text{max}}$) was not reached. The hyperbolic shape of the curves suggests that excited MB acts as a photocatalyst and that the relative absorbance change ($\Delta\Delta A / \Delta\Delta A_{\text{max}}$) was proportional to irradiance (I) and to the relative amount of unreacted material ($1 - \Delta\Delta A / \Delta\Delta A_{\text{max}}$) or:

$$\frac{\Delta\Delta A}{\Delta\Delta A_{\text{max}}} = K \cdot I \cdot \left(1 - \frac{\Delta\Delta A}{\Delta\Delta A_{\text{max}}}\right) \quad \text{where K is a constant}$$

Rearrangement yields

$$\frac{I}{\Delta\Delta A} = \frac{1}{\Delta\Delta A_{\text{max}}} \cdot I + \frac{1}{K\Delta\Delta A_{\text{max}}}$$

A plot of $I/\Delta\Delta A$ versus I results in straight lines for all combinations of I and MB (except where signal sizes were too small to be treated mathematically). This indicates that the formula adequately describes the I-dependence of $\Delta\Delta A$. It does not tell anything about the photochemical mechanism(s).

$\Delta\Delta A_{\text{max}}$ and K^{-1} could be calculated from the intercept on the ordinata and the slope of the lines. K^{-1} corresponds to the

irradiance needed for half maximal response and is thus a measure of the efficiency of the reaction. With increasing amount of MB, K^{-1} decreased, except at the highest MB concentrations. The same trend was also seen in the $\Delta\Delta A_{\max}$ obtained. The data also show that light cannot compensate for MB, since a smaller $\Delta\Delta A_{\max}$ was obtained if 12 μM MB was used, compared to the one obtained if a higher concentration was chosen.

For a photochemical reaction to occur an appropriate electron donor is needed. In flavin systems (Schmidt and Butler 1976) EDTA was used. This substance has been used in MB photo-reduction as well (Oster and Wotherspoon 1957). It was probably also the electron donor in the MB sensitized photo-reductions of the b-type cytochrome studied here, since omitting it from the resuspension medium led to much smaller LIACs.

Different oxygen species can be involved in flavin and MB sensitized photoreactions. Superoxide anions are involved in flavin mediated photoreductions (Ninnemann et al. 1977). The methylene blue mediated LIAC, did not seem to involve superoxide anions, since the addition of superoxide dismutase (20 $\mu\text{g ml}^{-1}$) did not affect the magnitude of the LIACs. This is also consistent with work by McCord & Fredovich (1970) in their study of the dark reduction of cyt c by ferredoxin-NADP reductase. Superoxide dismutase inhibited the reaction if it was mediated by flavins, but not if MB was used. Another oxygen species, namely singlet oxygen, has been implied in several MB sensitized photoreactions. It is probably not important in the photoreduction of b-type cytochromes, since the addition of sodium azide (5.4 mM), a singlet oxygen scavenger used in the study of several photodynamic processes (Kobayashi and Ito 1976), did not alter the LIACs obtained. Azide has other effects too, i.e. inhibition of flavin enzymes (Schmidt and Butler 1976) and binding to cytochrome a. A lack of importance of singlet oxygen was also observed in a study of the pH dependence. This revealed that a 10-30 % larger

LIAC was obtained at pH 7.0 than at 6.5 or 7.5, whether MB or thionine (a dye of the same family as MB) was used. The singlet oxygen production of these two dyes, has been shown to increase two-fold with a change of pH from 6.5 to 7.5 (Bonneau et al. 1975). Oxygen does play a role (perhaps in the reoxidation of MB, Usui et al. 1961), since in the respiring mitochondrial fraction (9 KP) the LIACs became irreversible if the cuvette was not aerated from time to time.

The pH dependence shown above also indicates that the rate limiting step in the reaction comes after the photoreduction of the dye. This is deduced, since Bonneau et al. (1973) showed that the quantum yield for photoreduction by EDTA of thionine dropped two-fold when the pH was increased from 6.5 to 7.5, contrary to a slight increase if MB was used. As mentioned above, no such differences were seen in the photoreduction of b-type cytochrome sensitized by thionine or MB.

A wavelength dependence study revealed that MB monomer was the active species, although the proportion of dimeric MB was substantial at the concentration chosen (seen as an absorption band around 610 nm). Only at high dye concentration did the dimer become active. This is consistent with its unreadiness to undergo photoreduction (Broyde and Oster 1959).

Addition of hydroquinone (3 mM) known to quench radicals (Cauzzo and Jori 1972), inhibited the light-induced cytochrome b reduction by 45 %. Radicals have also been proposed in flavin photochemistry (Schmidt and Butler 1976). Involvement of radicals in the reaction described here, does not prove that they reacted with the cytochrome. In a control experiment where leuco methylene blue (reduced by ascorbate) was added to the sample, cytochrome b reduction took place. Ascorbate alone did not reduce the cytochrome.

To sum up, methylene blue can substitute for the endogenous photoreceptor (probably a flavin) in light induced reductions

of a b-type cytochrome, although the photochemistry differs in some parts. The same type of discrimination between cytochromes is involved with a dominating activity in a fraction which presumably is enriched in plasma membranes. The photochemical step between the excited dye and the cytochrome is rate limiting and the pH optimum lies around 7.0.

3.2 Can the b-type cytochrome be purified completely without losing its LIAC activity? (III, IV and V)

The aim of this experimental series was to obtain a fraction where a similar difference spectrum was obtained whether light and MB on the one hand or dithionite on the other acted as reductant. Hopefully the similarity would lie both in the localization of the peak in the Soret region and in the magnitude of the $\Delta\Delta A$. A variety of separation methods were tested. A crude differential centrifugation as used above, only resulted in enrichments, i.e. no purity. For determination of the actual specificity between MB and the b-type cytochrome, a larger discrimination was needed. This was of interest since the cytochrome could serve as a membrane marker for plasma membranes in corn coleoptiles (no such marker has been found yet).

A balance sheet, of the total LIAC activity in all fractions compared to the amount before pelleting took place was made in order to compare the distribution of some membrane markers with that of LIAC. It was not possible to measure any reproducible LIAC in the supernatants so data from these could not be included. The membrane markers tested were cytochrome oxidase for mitochondria (Appelmans et al. 1955), NADH dependent antimycin A resistant cytochrome c reductase (Lord et al. 1973) for endoplasmatic reticulum and IDPase (Ray et al. 1969) for Golgi vesicles. They had the following relative distributions (relative to the fraction with most activity) between 9 KP : 21 KP : 50 KP : cyt oxidase 100 : 17 : 8,

cyt c reductase 16 : 32 : 100 and IDPase 77 : 10 : 83. The total recovery for the three enzymes were 121, 108 and 141 %, respectively. It is not known why larger recoveries than 100 % were obtained, but soluble substances interfered with the redox changes in cytochrome c. The MB sensitive cytochrome b was distributed between the fractions as 40 : 100 : 66, i.e. it did not coincide with either mitochondria or endoplasmatic reticulum as mentioned earlier. The differences in the IDPase activity between the fractions were not large enough for any conclusions to be drawn.

Detergents have been used in the purification of different cell components. Most of them are tightly bound to membranes (Kagawa 1974, Helenius and Simons 1975) and not possible to remove by salt or protein perturbants such as urea and chaotropic anions. The mechanism by which a detergent such as Triton X-100 attacks the specific membrane component is completely understood in only a few cases. Briefly, the steps are the following if the procedure takes place in water solution: At a very low concentration of detergent, some of the detergent molecules are dissolved, some from a monolayer on the water surface and some are bound to the membranes. When the detergent concentration is increased, a concentration is reached, above which no more individual molecules can be dissolved. This is called the critical micellar concentration (CMC). At still higher concentrations, large aggregates are formed with all lipophilic ends turned inwards. These aggregates are called micelles and as they are hydrophilic on the outside, they can be dissolved in water in large quantities. This means that instead of having a system where the detergent molecules were bound to membranes or free, the membranes are solubilized, and protein-detergent as well as lipid-detergent micelles are formed.

It was of interest to study the detergent effect on the distribution and activity of the MB sensitized photoreduction of cytochrome b. Detergents have been used in the purification of other b-type cytochromes. Cytochrome b_{559} high potential

from chloroplasts could be solubilized completely (100 %) with Triton X-100 if this was made 2 or 4 M with respect to urea (Garewal and Wasserman 1974) compared to 30 % solubilization if only Triton X-100 was used. Treatment with Tween 40 followed by Triton X-100 was effective in the solubilization of cyt b-561 from adrenal chromaffin granule membranes (Silsand and Flatmark 1974). Cytochrome b₅, the dominating cytochrome in the endoplasmatic reticulum has been investigated by means of detergents (Ito and Sato 1968, Spatz and Strittmatter 1971). All these cytochromes are tightly bound to the lipophilic interior of their respective membranes.

Four detergents were tested, namely the ionic ones sodium dodecyl sulphate and sodium deoxycholate and the non-ionic ones Triton X-100 and Tween 40. Sodium dodecyl sulphate was ruled out after the first experiments since it inhibited the LIAC. This effect was probably not due to MB binding to SDS as have been proposed by Usui et al. (1976) making it photo-dynamically inactive, at low EDTA concentrations (<10 mM), since photoreduction of MB took place. Rather, the effect could be ascribed to the denaturing capacity of SDS. Tween-40 in concentrations above CMC did not result in more than a minor solubilization of LIAC-related b-type cytochrome from the three pellets. This is not unexpected, since the detergents of the Tween series are not as lipophilic as Triton X-100 (Helenius and Simons 1975). Thus the LIAC is supposedly related to a cytochrome that is tightly membrane bound.

The two remaining detergents, i.e. DOC and Triton X-100 were both potent solubilizers for LIAC related cytochrome. Triton X-100 resulted in largest LIAC recovery. Cytochrome oxidase was also solubilized at least with Triton X-100 without loosing its accessibility to electrons from photo-reduced MB. A slightly higher detergent concentration was needed, however (1 %).

At the highest concentration used (1 % (w/w) given to samples with around 1 mg protein/ml) DOC acted inhibitory, contrary

to when Triton was used. If concentrations below CMC were chosen hardly anything was solubilized independent of the detergent used. DOC resulted in a large spectral shift of the 428 nm peak (to 418 nm) in the 9 KP but not in the two other fractions. Such an effect was not seen if Triton was chosen. It is not known whether the shift to 418 nm in DOC treated 9 KP was due to an activation of a cytochrome in the mitochondrial fraction, but the absorbance changes resemble those obtained upon c-type cytochrome reduction. In a control experiment reversible reduction of horse heart cytochrome c was obtained after irradiation with red light in the presence of MB. The reduction was abolished upon Triton X-100 treatment but not if DOC was added. This does not prove that c type cytochromes really were involved, since they ought to be seen in non-solubilized fractions as well. Besides, the spectra of cytochromes in detergent solution are not known. Neither has the accessibility to electrons from photoreduced MB for detergent treated cytochromes been studied. The solubilized material retained its LIAC maximum at 428 nm in the other fractions.

The recovery did not exceed 50 and 43 % respectively, in the 21 KP and 50 KP fractions, and was 70 % in the 9 KP. The reason for this loss of activity is not understood. It was checked that it was not a spectrophotometric error, due to pathlength elongation (the absorbance of a dye (riboflavin) was the same whether it was dissolved in buffer or sample). The loss of activity could indicate that the membrane environment is essential for optimal LIAC to occur. Sensitivity to detergents have been reported for other cytochromes, i.e. the high potential form of cyt b_{559} as mentioned earlier, and also cyt b_6 . In the former case the cytochrome could be preserved by introducing urea in the solubilization step. This medium was applied to membranes from corn coleoptiles, but the recovery increased only slightly. Neither was it improved if the easily soluble membrane components were extracted from the membranes by the use of Tween prior to Triton X-100, as was suggested by Silsand and Flatmark (1974)

in their work with cytochrome b-561.

The larger recovery in the fractions sedimenting at low g-values is consistent with the existence of two populations located in different membrane fractions and with unequal sensitivity to detergents. It could also be due to an effect analogous to that shown for nitrate reductase by Klemm and Ninnemann (1979) in their work with *Neurospora*. They found that this soluble flavohemoprotein lost its activity upon starvation concomitant with the appearance of blue light induced conidiation as well as cytochrome b reduction. At the same time, the activity of one of the subunits of nitrate reductase increased. The increased activity was associated with plasma membranes and mitochondria. Starvation could be substituted by acetone treatment. The detergent effect observed by me, can be compared with their acetone effect, at least in some respects, since both agents lyse membranes.

The nitrate reductase related b-type cytochrome is probably not the only hemoprotein that, at least under certain conditions, can accept electrons from photoreduced MB. In order to clarify this, purer fractions were needed. Therefore, 21 KP and 50 KP of a corn coleoptile preparation were collected together and then laid on a discontinuous sucrose gradient (15/28/33/45/ % w/w sucrose) which was run at 115 000 g for 5 hours. The material at each interface was collected in separate fractions. Assays were made for the same membrane markers as used above, before solubilization took part. LIAC as well as the amount of dithionite reducible cytochrome (measured as the "dithionite reduced" minus "no addition" absorption difference in the Soret region) was measured both before and after solubilization. The distribution of LIAC did not coincide with any of the markers as shown earlier in crude differential centrifugation. Most light induced cytochrome reduction was found in a fraction collected at the 33/44 % sucrose interface. As much as 40 % of the cytochrome in this fraction was reducible by photoreduced MB. Upon detergent treatment, similar decreases in LIAC activity as shown above, were

obtained. The total amount of cytochrome was not altered much, except in the 33/45 % sucrose interface fraction, indicating either a reduction or a destruction of the b-type cytochrome. In redox potential terms, this would correspond to an increase rather than a decrease. The parallelism to the high potential form of cytochrome b_{559} is probably therefore not valid any longer. A comparison between the dithionite reducible cytochromes before and after solubilization with the amount of LIAC revealed that if the total amount of cytochromes in detergent treated fractions were corrected for what is lost due to the postulated detergent induced cytochrome b reduction (or destruction) almost the same values as obtained prior to solubilization were obtained. (The comparison is valid only after the approximation that the reduced minus no addition absorption difference is proportional to cytochrome concentration also before solubilization.) A slight gain was the result for the 9 KP fraction (6 %) and a loss was seen in the fraction at the 33/45 % sucrose interface fraction (14 %). In the two other fractions only small changes were seen (2-3 %). This means that such detergent induced cytochrome reduction or destruction could take place or at least that the cytochrome cannot become reoxidized if once reduced.

It is probably not the whole answer, since it does not explain the shift to denser fractions collected at higher sucrose concentrations also seen in this kind of separation (not unexpected since both differential and sucrose gradient centrifugation separate the particles primarily due to their sizes). The dominating b-type cytochrome in the endoplasmatic reticulum was not involved in these light induced absorbance changes, since the LIAC in the 15/28 % sucrose interface fraction was small before solubilization and even smaller afterwards. The problem seems to lie in the fraction at the 33/45 % sucrose interface. Sucrose gradients not a discriminating enough for separation of plasma membrane from out roots mitochondria (Hodges and Leonard 1974). The same seems to be true here. Therefore methods based on other

properties had to be adopted.

Albertsson (1971) has developed methods for separation of particles based on their partition between two different phases. Here, the distribution is dependent on surface properties rather than particle sizes. He has shown that in a mixture of different polymers, for example PEG and dextran in low concentrations in water two phases will be formed (if the concentrations are not too low). The upper one is enriched in PEG and the lower one in dextran. If slightly higher concentrations are chosen, the respective enrichments will be larger. The top phase is slightly more hydrophobic than the bottom phase. When biological material is added to such a system, the individual components partition between the two phases according to their hydrophilic properties. It should be pointed out that both phases contain mainly water and thus are mild solvents for the biological material.

This seemed to be a suitable method in the separation of LIAC activity from mitochondrial contamination. First, a series of polymer concentrations were tested. Almost all the material appeared in the top phase when the phase system was 5.5 % (w/w) in both dextran T 500 and PEG 4 000. At concentrations of 6.5 % (w/w) almost everything appeared in the bottom phase. If an intermediate concentration was chosen, i.e. 6.1 % (w/w) (which results in a top phase with ~8 % PEG and <1 % dextran and a bottom phase with 16 % dextran and 2 % PEG), almost half of the LIAC activity appeared in the top phase compared to less than a fifth of that of cytochrome oxidase. Thus, cytochrome oxidase partitions with a larger preference for the bottom phase than the LIAC responsible b-type cytochrome. Therefore, this composition of phase system was chosen for further studies. Both top phase and bottom phase were transferred to new bottom and top phases, respectively (tubes 2 and 3), and carefully mixed. Table 1 shows the distribution of activity in the different fractions thus obtained.

Table 1

The distribution of cytochrome oxidase and LIAC in 21 KP + 50 KP from corn coleoptiles separated into different fractions with a two phase polymer system (6.1 % (w/w) PEG 4 000, 6.1 % (w/w) dextran T 500, 0.25 M sucrose and 5 mM potassium phosphate buffer; pH 7.8), at 5°C. The result is the mean of two experiments where 15 g coleoptiles were used.

	Fraction	Cytochrome oxidase			LIAC		
		$\Delta\Delta A_{\text{min}}^{-1}$	% top phase	% of total activity	$\Delta\Delta A \times 10^3$	% top phase	% of total activity
	T_2	0.66	16	3.5	18.3	55	27
	T_2B	3.4		20	14.7		21
	TB_2	2.4	16	12	9.4	27	13
	B_2	12.5		66	25.4		37

In tube 2, i.e. when the original material in the top phase had been purified once 55 % of the activity of the LIAC was found in the top phase (T_2) contrary to the distribution in tube 3, where only 27 % are found in the top (TB_2). In the case of cytochrome oxidase, however, similar distributions were found in the two tubes (16 %). Therefore, possibly two populations of cytochrome sensitive to red light in the presence of MB exist. Both of them have Soret peaks at 428 nm. The top phase T_2 , which is colourless, is probably enriched in plasma membranes (Christer Larsson, personal communication). Plasma membrane vesicles turned inside out would theoretically

appear in the bottom phase, but these are not as frequent as right side out vesicles (Christer Larsson, personal communication). Therefore other cytochromes are probably involved in the LIAC here. They could reside in the proplastids, since the bottom phase B₂ is enriched in these organelles (as judged by the yellow colorization by carotenoids).

The total amount of cytochrome in the top phase T₂ (dithionite reducible) was of the same order and had a similar difference spectrum as the cytochrome sensitive to photoreduced MB. In contrast, only 10 % of the cytochrome were sensitive in the bottom phase.

3.3 How is MB sensitized cytochrome b photoreduction distributed in other organs and organisms? (V)

It has not been resolved whether the b-type cytochrome in corn coleoptiles is related to phototropism or not. Photochemical reactions between flavins and cytochromes could be produced artificially (Schmidt and Butler 1976). This has been an argument against any relationship with physiology, since the cell contains many flavoproteins that readily undergo photoreduction upon irradiation with blue light. Lipson and Presti (1977) showed that blue light could induce b-type cytochrome reduction in many organisms, including HeLa cells. They estimated the quantum yield for the reaction in *Phycomyces* to be very low, i.e. 0.015. Their conclusion was that with such a low quantum yield, a connection to physiological responses could be ruled out. Their calculation method was tested on the MB-cytochrome system described here in corn coleoptile fractions as well as on the blue light induced cytochrome reduction in *Neurospora* (Britz et al. in preparation). About 50 times larger values were obtained, i.e. the process seems to be effective. The reason for the discrepancy between their result and ours is not understood. Some of the studies presented above indicate that more than one population of b-type cytochrome molecules resulted in

LIAC. Theoretically, it could be that only one of them, i.e. the one bound to plasma membranes was the one involved in phototropism, whereas the other were related to other enzymatic processes. A separation of membrane fragments from other plant sources (with different b-type cytochromes) could perhaps answer this question.

The study of b-type cytochromes is haphazardous, since they are involved in energy transduction in several cases. At least b-type cytochromes from animals have the capacity of changing both redox potential and absorption spectra (Slater and Lee 1973). Low potential forms, i.e. cytochromes reducible only by dithionite and without known physiological role and with Soret peaks at 428 nm have been found in plant mitochondria (Lambowitz and Bonner 1974). In mung beans, the alpha band differs from that of the b-type cytochrome in our study. In potatoes, two components could be seen, one of them being spectrally similar to our LIAC related cytochrome. In both plants the concentration of such low potential species was as great as that of our cytochrome. Several low potential cytochromes also belong to the microsomal fraction (20 000 - 40 000 g, 3 h) as shown by Rich and Bendall (1975). They made a survey of the distribution of such cytochromes in different plants. In cauliflower as well as in mung beans the Soret peaks coincided with that of the LIAC, whereas the alpha bands were shifted to slightly longer wavelenghts. In cauliflower, the components reducible by dithionite only, were probably mainly cytochromes P-450 and P-420 (as judged by CO-binding) whereas these as well as others were found in mung beans. In barley shoots, only a very small fraction could be attributed to CO-binding b-type cytochromes. Galston et al. (1978) reported on blue light induced cytochrome reductions in a microsomal fraction from peas (50 KP), also similar to the b-type cytochrome in our study.

Cauliflower inflorescences, barley shoots and corn shoots were chosen as objects for membrane separation with two phase polymer systems. Table 2 shows the distribution of cytochromes

Table 2

The distribution of cytochrome oxidase, LIAC and the ratio between LIAC and the total amount of dithionite reducible cytochrome (LIAC/Dt) in 21 KP + 50 KP from corn shoots, barley shoots and cauliflower inflorescences, separated into different fractions with a two phase polymer system (6.1 % (w/w) PEG 4 000, 6.1 % (w/w) dextran T 500, 0.25 M sucrose and 5 mM potassium phosphate buffer; pH 7.8 at 50°C). The activities are calculated to show the total activity with an initial fresh weight of 15 g. The tubes and fractions are named as in Table 1. n = number of experiments (from which a mean is calculated).

	Fraction	Cytochrome oxidase			LIAC			LIAC/Dt
		$\Delta\Delta A \text{ min}^{-1}$	% top phase	% of total activity	$\Delta\Delta A \times 10^3$	% top phase	% of total activity	
Corn shoots n = 1	T ₂	2.9	23	2.5	23	58	25	0.78
	T ₂ B	9.5		8	17		18	0.20
	TB ₂	14.3	14	12	22	42	24	0.27
	B ₂	88.0		77	31		33	0.09
Barley shoots n = 2	T ₂	4.8	51	12	25	71	36	0.45
	T ₂ B	4.7		11	10		14	0.19
	TB ₂	5.8	19	14	17	50	25	0.26
	B ₂	25.6		62	17		25	0.24
Cauliflower inflorescences n = 3	T ₂	1.2	22	3.0	30	71	45	0.82
	T ₂ B	4.3		11	12		18	0.26
	TB ₂	3.5	10	9	12	48	18	0.31
	B ₂	30.4		77	13		19	0.10

between membrane fractions in a separation as described for corn coleoptiles. In corn and barley shoots (with proplastid cytochromes) and cauliflower inflorescences (with cyt P-420 and P-450) LIAC could be obtained. In both corn and cauliflower only a small fraction of the respiratory activity appeared in the top phase (T_2) compared to more than 75 % in the bottom phase (B_2). These data are similar to those obtained from coleoptiles. Part of the activity in T_2 could be related to mitochondria enclosed in plasma membranes, since the partitioning between T_2 and T_2B is slightly higher than the one between TB_2 and B_2 . (The much larger cytochrome oxidase activities in Table 2 compared to Table 1 are probably because different batches of cytochrome c were used.) In the case of LIAC, however most of the activity was found in the respective T_2 and B_2 . Therefore, both plant materials contain more than one b-type cytochrome capable of being photoreduced by methylene blue. One of them is tentatively suggested to be bound to plasma membranes (enriched in the top phase). The LIAC related cytochrome dominated in the top phase (T_2) whereas it only contributed to 10 % of the bulk of cytochrome in the bottom phase B_2 . The same conclusions could probably also be drawn from the data of the barley preparation, even if the separation was not as good here. Moreover, the distribution of LIAC is the same whether cauliflower inflorescences or barley shoots were analyzed. This could indicate that cytochromes P-420 and P-450 were not involved in the LIAC. More data are needed to elucidate whether those cytochromes not related to plasma membranes (but able to produce LIAC) are the same as those in coleoptiles.

To sum up, similar LIAC could be found in corn and barley shoots and cauliflower inflorescences as was found in corn coleoptiles and also was reported from fungi. There are at least two types, one of them possibly bound to plasma membranes (as judged by phase partitioning). Whether this cytochrome is specifically related to photobiology or if it has another function as a component of an electron transport

chain used in other processes, e.g. ion uptake (Lundegårdh 1950) still remains to be studied.

3.4 Are there LIACs in the blue spectral region in dark grown coleoptiles, other than those related to cytochromes? (I and V)

When dark grown wheat coleoptiles, bundled together in a solution-filled cuvette were irradiated with blue light reversible as well as irreversible absorbance changes in the blue region were obtained. As was mentioned in the introduction, the discovery of the red far-red reversibility in phytochrome regulated processes initiated a search for, and led to the discovery of spectral changes upon irradiation. Are the blue light induced absorbance changes necessarily related to plant blue light photophysiology? A closer examination, revealed that this was not the case. The difference spectrum obtained had absorbance increases at 440 (larger) and 600 nm (smaller) and a decrease at 420 nm (relative to 500 nm). Most effective light for causing these absorbance changes was 420 nm with a hump at 530-570 nm. The absorbance changes were not possible to evoke until the coleoptiles had been in the cuvette for half an hour. They could partially be inhibited by potassium iodide, indicating that triplet states were involved. It was tentatively suggested that b and c type cytochromes were involved in the photosensitizing process, although the action spectrum did not quite coincide with that of either pigment. Cytochromes have been shown to be involved in the phototaxis of the slime mold *Dictyostelium* (Poff and Butler 1974 b) and it has also been shown that iodide can quench cytochrome triplets (Vorkink and Cusanovich 1974). The difference spectrum was similar to one that would be obtained upon cytochrome oxidase reduction. It was somewhat unclear how oxidized cytochrome, ready to undergo photoreduction could still be present after the coleoptiles had been in the cuvette for half an hour. (The oxygen tension was not measured.) An experiment indicating that

something also could be accumulated was, that the absorbance changes were not obtained in a flow cuvette, although the flowing liquid had been bubbled with nitrogen. The absorbance changes appeared again when the flow was stopped.

The same kind of absorbance changes (with a reversible large increase at 440 nm and a reversible as well as an irreversible decrease at 420 nm and also some smaller irreversible components at 530-580 nm as well as at reversible increase around 600 nm) were also found in other chlorophyll-less plants e.g. corn coleoptiles, corn mesocotyls, wheat roots and horseradish roots. Corn mesocotyls were chosen for sub-cellular localization of the pigment system. No activity was found in any of the fractions. This was expected, since the activity in whole organs did not appear unless air was excluded. After addition of dithionite, however, to the fractions, similar light induced absorbance changes were found. Since such a strong reductant could be used, together with the fact that for most activity was found in the supernatant after 50 KP, the tentative hypothesis including respiratory cytochromes, could totally be ruled out.

Lundegårdh (1951) had described a similar system in wheat roots. He found a cytochrome-like pigment, which he called cytochrome dh, which gave rise to absorption bands only after prolonged anaerobiosis in a non-streaming medium. Later (1958) he thought this pigment to be a peroxidase instead. If hydrogen peroxide was added to reduced horseradish peroxidase a spectrum similar to the one obtained by Lundegårdh as well as to the action spectrum for the light induced absorbance changes was obtained.

Recently, the same photochemical events have been described by Schneider and Bogorad (1978) in organs as well as cell-free extracts. They also made the comparison with peroxidases, and suggested that similar mechanisms as described in the reaction between peroxidase and NADH was involved (Yokota and Yamazaki 1965). Here, an enzyme complex with H_2O_2 , called compound III was formed, upon the oxidation of NADH. When

all NADH was consumed, the complex was split into a mixture of ferro- and ferrihemoproteins. The difference spectrum for this splitting process, would be similar to the one obtained upon irradiation of organs as well as plant extracts. Therefore, Schneider and Bogorad indicated that mechanisms similar to a light induced dissociation of compound III (or analogues) could be involved. They also drew the attention to the fact that peroxidases are known to form CO-complexes, which are split upon irradiation (Yamazaki et al. 1970).

Hemoproteins have been shown to be photosensitizers in several phenomena in plants as well as in animals. Respiration is inhibited due to bleaching of cytochromes (Epel 1973). Porphyrin in man is deleterious, since reactive porphyrin species are formed in the sun (Runge 1972). Proto-tetrahydroporphyrin IX, a precursor to cytochromes, peroxidases, etc. has also been shown to be light sensitive (Poulson and Polglase 1973).

There are also cases, where wavelengths other than blue and red has significant effect on physiology. The growth of tissue cultures of plant cells as well as HeLa cells, studied by Klein and Edsall (1967) was inhibited by 365 nm and green yellow light (530-570 nm). In the study of root geotropism in water cress, Klein (1979) showed that 550 nm light decreased the cell elongation on the upper side of the root (i.e. inhibited the geotropic response). This decrease could be negated by prior irradiation with red light (620). It could not be deduced which pigment system was involved.

In conclusion, while studying the absorbance changes that can be obtained in the blue part of the spectrum, one has to take into account that other pigments, besides cytochromes and flavins absorb in this region and are hence capable of undergoing light-induced absorbance changes. The action spectra for these changes cannot be related to physiological photoreceptors. It can not be ruled out, however, that they reflect intermediates in accepted photoreactions or that they have shifted peaks due to screening pigments.

4 EPILOGUE

The b-type cytochrome described in this dissertation was proposed to be involved in blue light photophysiology. Arguments have been raised, against such an interpretation. The data presented here indicate that there is a membrane fraction that almost lacks pigments other than this particular cytochrome. Based on surface properties as well as the lack of coincidence with membrane markers for mitochondria, Golgi and endoplasmatic reticulum a correlation to plasma membranes can probably be made. Hopefully, a future study as regards the distribution of this particular cytochrome in different plants will partially solve the problem whether it has a physiological significance. A more detailed study of the photochemistry in the different cases will also be needed before any photophysiological conclusions can be drawn.

Finally, it should be satisfying if MB given to coleoptiles and subsequent irradiation with red light would make the plants bend. Such experiments have not been successful, but the problem could lie in difficulties in MB-penetration of membranes. It is also possible that the dye has to be bound to the membranes in order to function. This problem will be left for the future. The peroxidase-like photochemical changes described for wheat coleoptiles and corn mesocotyls etc. also needs further research in order to be ruled out or accepted as part of important photoregulation mechanisms in plants.

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